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STATISTICAL OPTIMIZATION OF PROCESS VARIABLES USING CENTRAL COMPOSITE DESIGN FOR ENHANCED L-METHIONINASE PRODUCTION BY *ASPERGILLUS FLAVIPES*

A.SWATHI[1] & V. SRIDEVI [2]

[1] Research Scholar, Centre for Biotechnology, Department of Chemical Engineering, Andhra University College of Engineering (A), Andhra University, Visakhapatnam, Andhra Pradesh, India

[2]Professor, Centre for Biotechnology, Department Of Chemical Engineering, Andhra University College of Engineering (A), Andhra University, Visakhapatnam, Andhra Pradesh, India.

ABSTRACT

Response Surface Methodology (RSM) based on 4 factor Central Composite Design (CCD) was employed to obtain the best possible combination of process variables for enhanced L-methioninase production in solid state fermentation (SSF) by *Aspergillus flavipes*. These variables include inoculum volume, initial moisture content, carbon supplement concentration and pH. The experimental data obtained was fitted to a second-order polynomial equation using multiple regression analysis and analyzed by Analysis of Variance (ANOVA). 3D response surface plots derived from the mathematical models were applied to ascertain the optimal conditions. The model came out to be highly significant and the statistical analysis results showed that the interaction of medium components were strong. The predicted optimum levels were as follows: inoculum volume - 3.0814 ml, initial moisture content - 62.19003% (v/w), carbon supplement concentration - 0.9995% (w/v) and pH - 8.36124. Under these optimum conditions, the experimental yield of L-methioninase was 397 U/gds, which was in close agreement with the value predicted by the model, 396.5811 U/gds. The value of regression coefficient R^2 = 0.9874, indicated that 98.74% of the variability in the response could be explained by the model. The enzyme L-methioninase was partially purified using 70% saturated ammonium sulphate followed by dialysis. The purification fold increased from 1 in the crude extract to 2.606 in the dialyzed enzyme sample.

KEYWORDS: Central Composite Design, L-Methioninase, Optimisation, *Aspergillus Flavipes*, Solid State Fermentation, Purification

INTRODUCTION

The use of enzyme as drug is a crucial facet of today's pharmaceutical industry. L-methioninase (E.C 4.4.1.11), a potent anticancer agent, is a pyridoxal phosphate dependent enzyme that catalyzes the deamination and demethiolation of L-methionine to α-ketobutyrate, methanethiol and ammonia (Ruiz-Herrera and Starkey, 1969). L-methioninase has antioxidant activity and downregulates the amount of polyamines (Moinard *et al*., 2005; Gilmour, 2007) and it improves the aroma of foods by degradation of L-methionine, which releases volatile sulphur compounds (Cuer *et al*., 1979). Normal cells have the ability to grow on homocysteine, instead of methionine, due to their active methionine synthase (Mecham *et al*., 1983). Unlike normal cells, tumor cells are devoid of active methionine synthase and they depend on external methionine supplementation (Hoffman, 1984). Methionine-dependency was reported in colon, kidney, prostate, melanoma, and fibrosarcoma tumor cells (Yamamoto *et al*., 2003).

L-Methioninase is ubiquitous in all organisms except in mammals. L-methioninase production by fungi including *Microsporum gypseum*, *Scopulariopsis brevicaulis* (Stahl *et al*., 1949) *Aspergillus* sp. Rs-1a (Ruiz-Herrera and Starkey, 1969), *Debaromycs hanseni* (Bonnarme *et al*., 2001) and *A. flavipes* (Khalaf and El-Sayed, 2009; El-Sayed, 2009) was studied. L-methioninase was purified and characterized from solid cultures of *A. flavipes* using chicken feathers (El-Sayed, 2011). The search for novel producers for large scale production of this enzyme at low cost has become a challenge because of its highly therapeutic properties. Solid-state fermentation (SSF) on agro-industrial residues promises a cost-effective bioprocess with higher yields (Pandey *et al*., 1999). In SSF, the yield of the enzyme can be improved by manipulating the nutritional and physical parameters (Gohel *et al*., 2006).

Response surface methodology (RSM) consists of a group of statistical techniques for developing empirical model and its exploitation. It defines the effect of the independent variables, alone or in combination, on the process. In addition, this experimental methodology generates a mathematical model that accurately describes the overall process (Li *et al*., 2007). Through a careful design and analysis of experiments, RSM try to relate a response (or output variable), to the levels of a number of parameters (or input variables) that affect such response.

In this article, we have reported a sequential optimization strategy for L-methioninase production by *Aspergillus flavipes* in SSF through statistically designed experiments. First, one-variable-at-a time screening design was applied to address the most significant variables affecting L-methioninase production. Secondly, a CCD was used to describe the nature of the response surface in the experimental region, to search optimal medium composition for maximizing L-methioninase yield. To the best of our knowledge, this was the first article describing the statistical optimization of L-methioninase production by *A. flavipes* in SSF.

MATERIALS AND METHODS

Microorganism and Culture conditions

Aspergillus flavipes MTCC 6337, used in this study was procured from Microbial Type Culture Collection and Genebank, Chandigarh, India. The culture was maintained on Czapek's Yeast extract Agar (CYA) medium at 25 ºC for 5 days and stored at 4 ºC. Sub-culturing was done monthly.

Inoculum Preparation

Inoculum was prepared by adding 10 ml of sterile water containing 0.1% Tween-80 and then scraping the surface of sporulating slants (5 d old cultures of *A. flavipes*). Each ml of suspension contained 10^5–10^6 spores as counted by haemocytometer.

SSF Medium

Sesame oil cake, an agro-industrial residue, obtained from local oil mill was used as the solid substrate for L-methioninase production. 5 g of sesame oil cake was supplemented with 10 ml of salt solution containing 1.0% glucose, 0.25% KH_2PO_4, 0.05% KCl and 0.05 $MgSO_4 \cdot 7H_2O$ of pH 7.0. The conical flasks were sealed with hydrophobic cotton and autoclaved at 121 ºC for 20 min. The cooled substrate was inoculated with 1 ml inoculum suspension. The flasks were mixed thoroughly and incubated for 8 days at 28 ºC under static conditions (El-Sayed, 2009).

Enzyme Extraction and L-Methioninase Assay

The crude L-methioninase was extracted with 40 ml of 0.1 M phosphate buffer (pH 7.0), centrifuged at 4 ºC for

15 min at 10,000 rpm. The supernatant was used for enzymatic assay. Assay of methioninase was carried out by slight modification of Imada *et al*., (1973) using L-methionine as substrate. L-methionine (0.5 ml of 1 %) in 0.1 M phosphate buffer (pH 7.0) and 0.1 ml pyridoxal phosphate was made to react with 0.5 ml of methioninase enzyme for 1 h at 30 ºC. The reaction was stopped by adding 0.5 ml of 1.5 N trichloroacetic acid. Then 0.1 ml of above mixture was taken to which 3.7 ml of distilled water was added. Then 0.2 ml Nessler's reagent was added to it, the developed color was measured after 20 min at 480 nm using a spectrophotometer. One unit (U) of L-methioninase was defined as the amount of enzyme that liberates one µmole of ammonia/ hour under optimal assay conditions. Enzyme yield was expressed as the activity of the L-methioninase per gram dry substrate (U/gds).

Optimization Study for L-Methioninase Production

As a first optimization step, the critical variables for L-methioninase production along with their variation ranges were estimated based on classical 'one-variable-a-time' approach. The effect of inoculum volume (1-5 ml), moisture content (40-90% v/w), carbon supplement (glucose) concentration (0.2-1.4% w/v), and pH (6-12) were investigated. All experiments were conducted in at duplicate, and the mean values were calculated.

The central composite design was conducted in the optimum vicinity to locate the true optimum concentrations for the 4 factors which show significant effect on L-methioninase production. Twenty six experiments were conducted with 16 factorial points (2^4), 8 axial points (2×4) and 2 replications at the centre points (n_o= 2) according to CCD and L-methioninase yield was measured in each case. The range and centre point values of 4 independent variables were based on the results of preliminary experiments. The second-order polynomial coefficients were calculated to determine the role of each variable, their interactions and statistical analysis to obtain predicted yield of L-methioninase.

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{14}X_1X_4 + \beta_{23}X_2X_3 + \beta_{24}X_2X_4 + \beta_{34}X_3X_4 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 + \beta_{44}X_4^2$$

where Y is the predicted response, β_0 is the intercept, β_1, β_2, β_3 and β_4 are the linear coefficients, β_{11}, β_{22}, β_{33} and β_{44} are the squared coefficients, β_{12}, β_{13}, β_{14}, β_{23}, β_{24} and β_{34} are the interaction coefficients and X_1, X_2, X_3, X_4, X_1^2, X_2^2, X_3^2, X_4^2, X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_2X_4 and X_3X_4 are independent variables. Using 'Statistica 6.0' software, the data obtained was analyzed and response surface plots were constructed which indicated the possibility for enhancement in the production of L-methioninase. Statistical analysis of the model was performed to evaluate the Analysis of Variance (ANOVA).

Partial Purification of L-Methioninase

70%, 75% and 80% saturation of ammonium sulphate solution was added slowly by constant stirring to test tubes containing 5 ml of crude extract. The mixture was left for 12 h at 4 ºC, followed by centrifugation at 10,000 rpm for 20 min at 4 ºC. The precipitate was dissolved in 0.1 M phosphate buffer of pH 7.0 and dialyzed overnight against the same buffer at 4 ºC. The dialysis step was repeated until the complete removal of ammonium sulfate, as verified by barium chloride.

Protein Estimation

The enzyme protein content was determined by the method of Lowry *et al*., (1951), using bovine serum albumin as the standard.

RESULTS AND DISCUSSIONS

Optimization of Process Parameters Using CCD

The optimum conditions for L-methioninase production obtained in preliminary studies were - inoculum volume (3 ml), moisture content (60% v/w), carbon supplement (glucose) concentration (1% w/v), and pH (8) were obtained. These optimum values were used as the basis for selecting the mid points (zero level) in CCD (Table 1) for further optimization.

Table 1: Coded and Real Values of Medium Components Used for CCD

Independent Variables	Coded Factors				
	-2	-1	0	1	2
Inoculum volume (ml) (X_1) (Step =1)	1	2	3	4	5
Initial moisture content (% v/w) (X_2) (Step = 10)	40	50	60	70	80
Carbon supplement concentration (% w/v) (X_3) (Step = 0.2)	0.6	0.8	1.0	1.2	1.4
pH (X_4) (Step = 1)	6	7	8	9	10

Experiments were performed according to the CCD, given in Table 2 in order to evaluate the optimum combination of selected components in the medium.

Table 2: Result of CCD for 4 Factors and Comparison of Experimental and Predicted Values of L-Methioninase Yield

Run No.	Inoculum Vol. (Ml) (X_1)	Moisture Content (% V/W) (X_2)	Carbon Supplement (% W/V) (X_3)	Ph (X_4)	L-Methioninase Yield (U/Gds) (Y)		
					Experimental	Predicted	Residual
1	2 (-1)	50 (-1)	0.8 (-1)	7 (-1)	375.2700	374.9725	0.297500
2	2 (-1)	50 (-1)	0.8 (-1)	9 (1)	377.1500	377.1996	-0.049583
3	2 (-1)	50 (-1)	1.2 (1)	7 (-1)	372.1600	372.5629	-0.402917
4	2 (-1)	50 (-1)	1.2 (1)	9 (1)	377.7500	3777725	-0.022500
5	2 (-1)	70 (1)	0.8 (-1)	7 (-1)	375.6400	376.0913	-0.451250
6	2 (-1)	70 (1)	0.8 (-1)	9 (1)	378.9500	378.8908	0.059167
7	2 (-1)	70 (1)	1.2 (1)	7 (-1)	375.1900	374.8592	0.330833
8	2 (-1)	70 (1)	1.2 (1)	9 (1)	380.6600	380.6413	0.018750
9	4 (1)	50 (-1)	0.8 (-1)	7 (-1)	375.5000	375.8263	-0.326250
10	4 (1)	50 (-1)	0.8 (-1)	9 (1)	376.9800	376.9758	0.004167
11	4 (1)	50 (-1)	1.2 (1)	7 (-1)	373.0000	372.9758	0.275833
12	4 (1)	50 (-1)	1.2 (1)	9 (1)	377.0000	376.8563	0.1437500
13	4 (1)	70 (1)	0.8 (-1)	7 (-1)	381.5400	381.1825	0.257500
14	4 (1)	70 (1)	0.8 (-1)	9 (1)	383.0000	382.9046	0.095417
15	4 (1)	70 (1)	1.2 (1)	7 (-1)	379.0000	379.2579	-0.257917
16	4 (1)	70 (1)	1.2 (1)	9 (1)	384.0000	383.9625	0.037500
17	1 (-2)	60 (0)	1.0 (0)	8 (0)	365.0700	364.9463	0.123750
18	5 (2)	60 (0)	1.0 (0)	8 (0)	368.9700	369.1213	-0.151250
19	3 (0)	40 (-2)	1.0 (0)	8 (0)	371.9100	371.8562	0.053750
20	3 (0)	80 (2)	1.0 (0)	8 (0)	380.0000	380.0812	-0.081250
21	3 (0)	60 (0)	0.6 (-2)	8 (0)	382.0000	381.9796	0.020417
22	3 (0)	60 (0)	1.4 (2)	8 (0)	383.5800	380.6279	-0.047917
23	3 (0)	60 (0)	1.0 (0)	6 (-2)	383.0000	382.8979	0.102083
24	3 (0)	60 (0)	1.0 (0)	10 (2)	389.7000	389.8296	-0.129583

Table 2 cond.,							
25	3 (0)	60 (0)	1.0 (0)	8 (0)	395.0000	395.0000	0.000000
26	3 (0)	60 (0)	1.0 (0)	8 (0)	395.0000	395.0000	0.000000
27	3 (0)	60 (0)	1.0 (0)	8 (0)	395.0000	395.0000	0.000000
28	3 (0)	60 (0)	1.0 (0)	8 (0)	395.0000	395.0000	0.000000
29	3 (0)	60 (0)	1.0 (0)	8 (0)	395.0000	395.0000	0.000000
30	3 (0)	60 (0)	1.0 (0)	8 (0)	395.0000	395.0000	0.000000

Using the results of the experiments, the following second order regression equation giving L-methioninase yield as a function of inoculum volume (X_1), moisture content (X_2), carbon supplement concentration (X_3) and ph (X_4) was obtained.

$$Y = -67.5962 + 41.1575\,X_1 + 5.6355\,X_2 + 145.9542\,X_3 + 36.4992\,X_4 + 0.1059\,X_1X_2 - 0.8656\,X_1X_3 - 0.2694\,X_1X_4 + 0.1472\,X_2X_3 + 0.0143\,X_2X_4 + 3.7281\,X_3X_4 - 7.2416\,X_1^2 - 0.0501\,X_2^2 - 91.8516\,X_3^2 - 2.4091\,X_4^2 \quad (1)$$

The estimated coefficients along with their p-values were reported in Table 3.

Table 3: Regression Data for the Model

	Coefficient	Regression	Std .Error	T-Value	P-Value
Constant	β_0	-67.5962	6.761855	-9.997	0.000000*
X_1	β_1	41.1575	0.803475	51.224	0.000000*
X_1^2	β_{11}	-7.2416	0.050672	-142.911	0.000000*
X_2	β_2	5.6355	0.089668	62.849	0.000000*
X_2^2	β_{22}	-0.0501	0.000507	-98.829	0.000000*
X_3	β_3	145.9542	4.299634	33.946	0.000000*
X_3^2	β_{33}	-91.8516	1.266792	-72.507	0.000000*
X_4	β_4	36.4992	0.984052	37.946	0.000000*
X_4^2	β_{44}	-2.4091	0.050672	-47.543	0.000000*
X_1X_2	β_{12}	0.1059	0.006634	15.968	0.000000*
X_1X_3	β_{13}	-0.8656	0.331724	-2.609	0.019723*
X_1X_4	β_{14}	-0.2694	0.066345	-4.060	0.001026*
X_2X_3	β_{23}	0.1472	0.033172	4.437	0.000486*
X_2X_4	β_{24}	0.0143	0.006634	2.157	0.047612*
X_3X_4	β_{34}	3.7281	0.331724	11.239	0.000000*

Significant* ($p \leq 0.05$)

The coefficients of the regression model (Eq. 1) calculated were listed in Table 3, in which they contain four linear, four quadratic, four interaction terms and one block term. The significance of each coefficient in equation (5.1) was determined by student's t-test and p-values which were also listed in Table 3. The larger the magnitude of the t-value and smaller the p-value, the more significant is the corresponding coefficient. The p-values were used as a tool to check the significance of each of the coefficients, which, in turn, are necessary to understand the pattern of the mutual interactions between the test variables (Khuri and Cornell, 1996). This implies that the linear, quadratic and interaction effects of inoculum volume, moisture content, carbon supplement concentration and pH were highly significant as is evident from their respective p-values.

The results of the second order response surface model fitting in the form of Analysis of Variance (ANOVA) were given in Table 4. The Fisher variance ratio, the F-value ($= S^2_r / S^2_e$) is a statistically valid measure of how well the factors describe the variation in the data about its mean. The greater the F-value is from unity, the more certain it is that the factors explain adequately the variation in the data about its mean, and the estimated factor effects are real. The ANOVA

of the regression model demonstrates that the model is highly significant, as is evident from the Fisher's F-test (F_{model} =2288.4) and a very low probability value (P_{model} > F= 0.000000).

Table 4: ANOVA for the Model

Source of variation	Sum of Squares (SS)	Degree of Freedom	Mean Square (MS)	F-Value	P> F
Factor	2255.447	14	161.1034	2288.4	0.000000
Error	1.056	15	0.0704		
Total	**2256.503**	**29**			

The goodness of the fit of the model was checked by the determination coefficient (R^2). The R^2 value provides a measure of how much variability in the observed response values can be explained by the experimental variables and their interactions. The R^2 value is always between 0 and 1. The closer the R^2 value is to 1, the stronger the model is and the better it predicts the response (Tanyildizi *et al*., 2005). In this case, the value of the determination coefficient (R^2 = 0. 9874) indicates that 98.74 % of the variability in the response could be explained by the model. In addition, the value of the adjusted determination coefficient (R^2_{adj} = 0.9812) is also very high to advocate for a high significance of the model.

Interaction Effects of Fermentation Variables

The yield of L-methioninase over different combinations of independent variables was visualized through three-dimensional view of response surface plots in Fig 1 to 6. Response surface plot is a function of two factors at a time maintaining all other factors at a fixed level (zero for instance) which is more helpful in understanding both the main and interaction effects of the two factors.

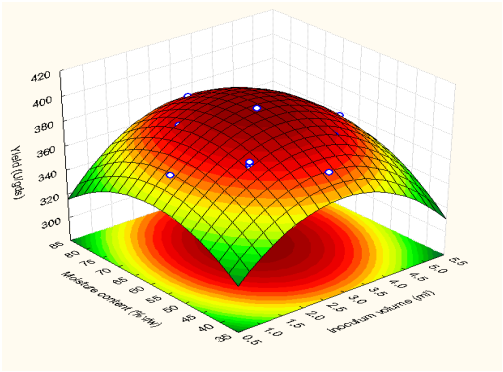

Figure 1: Response of inoculum. Volume vs moisture content

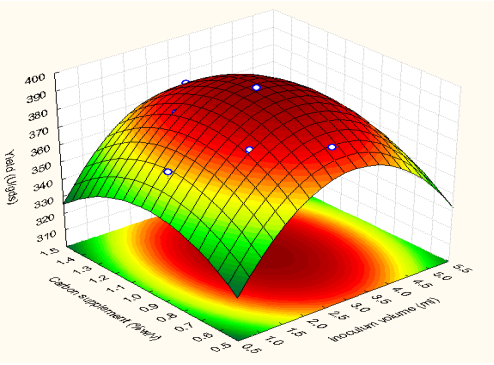

Figure 2: Response of inoculum volume vs. glucose concentrati

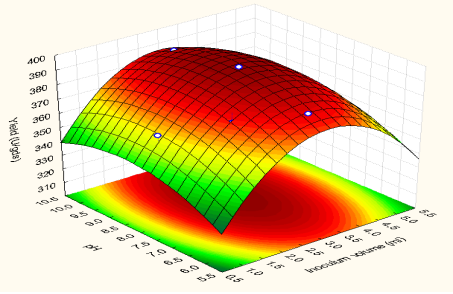

Figure 3: Response of Inoculum Volume Vs. pH

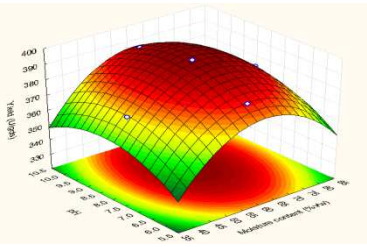

Figure 4: Response of Moisture Content Vs. pH

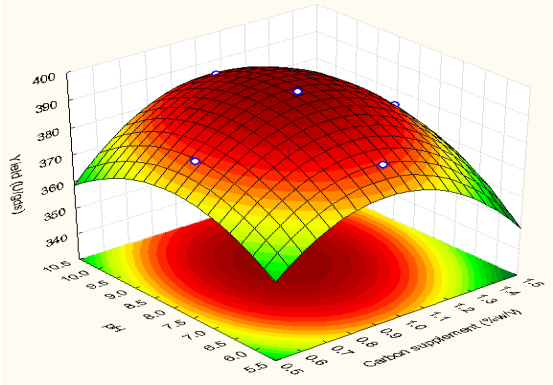

Figure 5: Response of Glucose Concentration Vs. pH

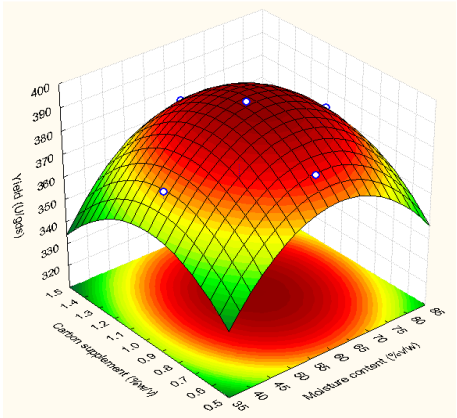

Figure 6: Response of Moisture Content Vs. Glucose Concentration

As clear from Fig. 1, 2, 3, 5, 6 the minimum response for enzyme production occurred when glucose concentration and inoculum volume both were in low concentration, while production increased considerably as concentration of glucose and inoculum volume were increased. This suggested the glucose concentration and inoculum volume have a significant effect on enzyme production.

Almost every biological process is pH dependent; a small variation in pH changes the enzyme yield. Hence, the optimal pH was very important for maximizing the yield of L-methioninase production. Fig. 3-5 showed the maximum and minimum pH responses on the L-methioninase production.

It was clear from Fig. 1, 4, 6 that an increase in the moisture content up to the optimum point increased the L-methioninase yield to maximum level.

Validation of the Experimental Model

Therefore, an optimum was observed near the central value of inoculum volume, moisture content, carbon supplement concentration and pH. The optimum conditions for maximum L-methioninase yield were obtained at inoculum volume of 3.0814 ml, initial moisture content of 62.19003% (v/w), carbon supplement concentration of 0.9995% (w/v) and pH of 8.36124. The predicted yield was 396.5811 U/gds. An experimental L-methioninase yield of 397 U/gds was obtained at these optimum parameters. The experimental values were found to be very close to the predicted values and hence, the model was successfully validated.

Partial Purification of L-Methioninase

After filtration of the fermentation broth, the filtrate was centrifuged at 5000 rpm for 15 min at 4 ^{0}C to obtain clear supernatant. The supernatant with L-methioninase activity of 49.625 (U/ml) and specific activity of 3070.85 (U/mg), was used as crude enzyme extract. The enzyme precipitation was carried out at different ammonium sulphate saturations such as 70%, 80%, 90%. The maximum enzyme precipitate was obtained at 70% ammonium sulphate saturation (Fig. 7) with a specific activity of 5538.5 (U/mg) which was equivalent to (1.804) purification fold. The precipitate was dissolved in minimal volume of potassium phosphate buffer of pH 7.0 and dialyzed against buffer. The dialyzed fraction (partially purified methioninase) had a specific activity of 8004.03 (U/mg) which was equivalent to (2.606) purification fold. The purification results were tabulated in Table 5.

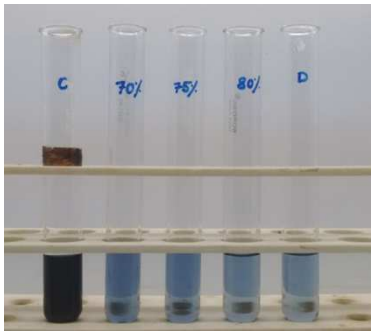

Figure7: Estimation of Protein Content by Lowry Method

Table 5: Partial Purification Profile of L-Methioninase from *Aspergillus flavipes* MTCC 6337

Purification Steps	Total Volume (Ml)	Total Enzyme Activity (U)	Total Protein Content (Mg)	Specific Activity (U/Mg)	Purification Fold	% Yield
Crude Extract	50	2481.25	0.808	3070.85	1	100
$(NH_4)_2SO_4$ Precipitation	25	1240.625	0.224	5538.5	1.804	50
Dialysis	20	992.5	0.124	8004.03	2.606	40

CONCLUSIONS

In this work, medium components for maximum L-methioninase production from *A. flavipes* were optimized by RSM. Central composite design was used to study the interactive effects of inoculum volume, moisture content, carbon supplement concentration and pH. The predicted optimum levels were as follows: inoculum volume - 3.0814 ml, initial moisture content - 62.19003% (v/w), carbon supplement concentration - 0.9995% (w/v) and pH - 8.36124. The results show a close agreement between the experimental and the predicted values. The enzyme was partially purified following techniques of ammonium sulphate precipitation and dialysis. The specific activity and purification fold increased considerably in the dialysis step compared to the crude extract. L-methioninase, an enzyme of super therapeutic and technological value could be sustained by conventional lower economical/higher efficiency productive bioprocesses.

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